I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Washington, DC 22313-1450, on the date shown below.

Dated: 12-13-06 Signature: Katherine L. Neville

Docket No.: 01017/36667
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Banks, W.

Application No.: 10/049,182

Group Art Unit: 1649

Filing Date: June 19, 2002

Examiner: Kolker, D.

For: Modulation of the Blood-Brain Barrier Transporter for Leptin

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. WILLIAM BANKS

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Dr. William Banks, do hereby declare and state as follows:

1. I am on the faculty at St Louis University in St Louis, MO, and am a U.S. citizen. I am an inventor of the invention claimed in the above-referenced application, and am providing this declaration to provide evidence in support of patentability of the invention.

2. In experiments performed in my laboratory to measure leptin transport across the blood brain barrier (BBB), the effects of the adrenergic agonists epinephrine and phenylephrine, the same agents as used in Borges et al. (*Eur. J Pharmacol.* 1994, 269:243-48), have been measured. As set out in the patent application, these experiments showed that epinephrine enhances leptin transport across the BBB. These experiments also demonstrated that phenylephrine does not increase transport of leptin across the BBB. See Figure 1 attached. Thus, the agents disclosed in Borges et al. as permeabilizers of the blood brain barrier do not all enhance the transport of leptin across the BBB.

3. Further studies of leptin transport across the BBB carried out in my lab, published in (Nonaka et al., *Brain Res.* 1016:58-65, 2004) (see Exhibit A of Response),

demonstrate that administration of the BBB permeabilizing agent LPS inhibited the transport of leptin across the Blood brain barrier. These studies illustrate that the transport of leptin across the BBB is through a specific mechanism and is not enhanced by general breakdown of the BBB.

4. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date 12/13/06

Dr. William Banks

Figure 1 of Declaration

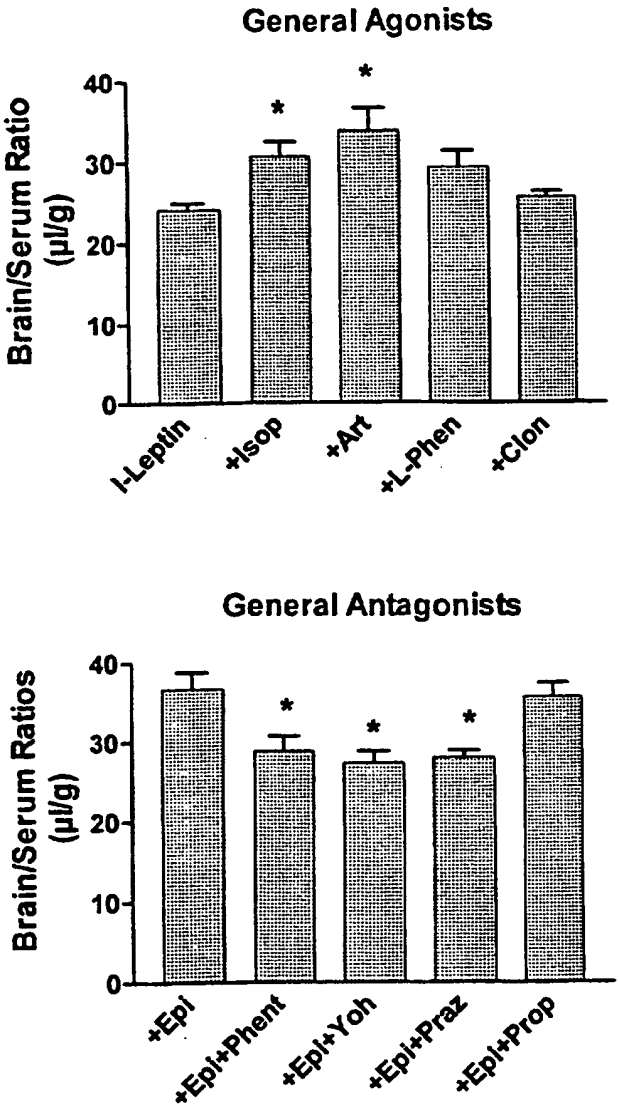

Fig. 6. Effect of general adrenergic receptor ligands on I-Lep uptake. Upper panel: effect of agonists on I-Leptin uptake. Isoproterenol and arterenol (norepinephrine) increased I-Lep uptake; L-phenylephrine and clonidine were without effect. * $P<0.05$ in comparison to I-Leptin value. Lower panel: Effect of antagonists on blockade of the epinephrine effect. Phentolamine, yohimbine, and prazocin blocked the effect of epinephrine on I-Lep uptake, but propranolol was without effect. * $P<0.05$ in comparison to '+Epi' group.

(Banks et al., Brain Res. 899:209-17, 2001)

Available online at www.sciencedirect.com

SCIENCE DIRECT

Brain Research 1016 (2004) 58–65

BRAIN RESEARCH

www.elsevier.com/locate/brainres

Research report

Effects of lipopolysaccharide on leptin transport across the blood–brain barrier

Naoko Nonaka[a,b,c,d], Stanley M. Hileman[e], Seiji Shioda[b], Thanh Q. Vo[c,d], William A. Banks[c,d,*]

[a] Department of Oral Anatomy, Showa University School of Dentistry, Tokyo, Japan
[b] Department of 1st Anatomy, Showa University School of Medicine, Tokyo, Japan
[c] Geriatric Research, Education, and Clinical Center, Veteran Affairs Medical Center-St. Louis, St. Louis, MO, USA
[d] Department of Internal Medicine, Division of Geriatrics, Saint Louis University School of Medicine, St. Louis, MO, USA
[e] Department of Physiology, West Virginia University, Morgantown, WV, USA

Accepted 22 April 2004
Available online 15 June 2004

Abstract

Leptin is a 16-kDa protein secreted by fat cells and transported into the brain where it decreases appetite and increases body temperature. Leptin transport is saturable and regulated by epinephrine, triglycerides, and starvation. Lipopolysaccharide (LPS) is derived from bacterial cell walls and also decreases appetite and increases body temperature. LPS is known to increase leptin levels in serum and to affect the passage of other regulatory proteins across the blood–brain barrier (BBB). Here, we examined the ability of LPS, at doses which induce weight loss, to modify the BBB transport of radioactive leptin (I-Lep). The transport rate of intravenously injected I-Lep was decreased by 50–60% from 8 to 12 h after a single i.p. injection of LPS (3 mg/kg). The effect of LPS was dose-dependent. In comparison to the brain/serum ratio, the baseline cerebrospinal fluid (CSF)/serum ratio for I-Lep was much lower and not inhibited by LPS. LPS did not affect I-Lep transport when studied by the brain perfusion method nor was Ob-Ra mRNA expression in isolated brain microvessels altered, demonstrating that a circulating factor rather than altered BBB function was responsible for inhibition. Brain perfusion showed that LPS was not this factor. Serum leptin was doubled and serum triglycerides increased by 44% after LPS administration, suggesting these to be the circulating inhibitory factors. In conclusion, a single dose of LPS has long-lasting effects on the transport of serum leptin across the BBB that are likely mediated through self-inhibition and triglycerides.
Published by Elsevier B.V.

Keywords: Mouse; Obesity; Protein; Appetite; CNS; Anorexia

1. Introduction

Leptin, cloned from the Ob gene [35], is a 16 kDa protein secreted from fat which regulates adiposity by decreasing feeding and increasing thermogenesis [9,20]. Leptin exerts these effects in the arcuate nucleus and probably elsewhere as its receptors are found throughout the brain [12,14]. Leptin enters the brain by way of a saturable transport system located at both the endothelium and choroid plexus [2,36]. Obesity in humans is associated with a resistance to leptin [11]. This resistance is probably caused by defects at the brain receptor, the blood–brain barrier (BBB), and downstream neuronal circuitries.

LPS, like leptin, decreases appetite and increases body temperature. Lipopolysaccharide (LPS) is derived from bacterial cell walls and induces a sickness behavior, primarily through the release of proinflammatory cytokines [26,27]. Leptin is one of the cytokines released by LPS [29,30] and it has been suggested that some of the effects of LPS, including those on anorexia and thermogenesis, are mediated through release of leptin [17,19]. Effects of LPS known to be mediated through leptin are protection against caerulein-induced pancreatitis [24] and release of interleukin-1 receptor antagonist [16,18].

LPS modifies the permeability of the BBB to albumin [33], insulin [34], and gp120 [3]. The question arises whether LPS can alter leptin transport across the BBB.

* Corresponding author. GRECC, Research Service, Veterans Affairs Medical Center,-St. Louis, 915 N. Grand Blvd., St. Louis, MO 63106, USA. Tel.: +1-314-289-7084; fax: +1-314-289-6374.
E-mail address: bankswa@slu.edu (W.A. Banks).

0006-8993/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.brainres.2004.04.066

The leptin transporter is modulated by other substances. Its activity is enhanced 2–3 fold by alpha 1-adrenergics and its rate is greatly decreased by triglycerides [7], obesity, and prolonged fasting or starvation [1,4,7,25]. The short form of the leptin receptor has been suggested to be either the leptin transporter [21] or a modulatory unit of the transporter [6]. In obesity, decreased transport of circulating leptin is primarily caused more by a reduced capacity of the leptin transport system than by saturation from the higher blood levels of leptin in obesity [4,5,28]. Recently, serum triglycerides have been identified as a major inhibitor of leptin transport [7].

Here, we gave injections of LPS at a dose which induces weight loss and sickness behavior to determine whether it could affect the transport of leptin across the BBB.

2. Methods

2.1. Radioactive labeling

Recombinant murine leptin (Amgen, Thousand Oaks, CA) was radioiodinated by the lactoperoxidase method. Briefly, 10 μg of leptin was mixed with 30 μl of 0.4 M sodium acetate (pH 5.6), 2.5 μl of lactoperoxidase, and 2 mCi of ^{131}I. The reaction was started by adding H_2O_2 in a volume of 2.5 μl; 10 min later, additional H_2O_2 in a volume of 2.5 μl was added. At the end of this second 10-min period, radioactively labeled leptin (I-Lep) was purified on a Sephadex G-10 column.

2.2. Measurement of BBB permeability to i.v. I-Lep

Methods previously used to examine I-Lep transport across the BBB were used [2,4,5]. Male ICR mice (2 mo old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40% solution). The left jugular vein and right carotid artery were then exposed. The mice were given an injection into the left jugular vein of 0.2 ml of lactated Ringer's solution (LR) containing 250,000 cpm of I-Lep. Blood was collected from the right carotid artery and the whole brain was removed and weighed 10 min after the i.v. injection. The pineal and pituitary were not included in the whole brain samples. The whole blood was centrifuged at 5400 × *g* for 10 min at 4 °C and the level of radioactivity measured in 50 μl of the resulting serum. The levels of radioactivity in serum and for whole brain was counted in a gamma counter for 3 min. The brain/serum ratio (μl/g) for whole brain was calculated by the formula:

Brain/serum ratio (μl/g)

= (cpm/g of brain)/(cpm/μl of serum).

In other mice, multiple-time regression analysis was used to more definitively separate the components of transport vs. reversible binding to the BBB. Mice were given an i.v. injection of I-Lep as above but brain and serum were collected at 1, 2, 3, 4, 5, 6, 7.5, 9, and 10 min later. Brain/serum ratios were plotted against exposure time (Expt):

$$\text{Expt} = \left[\int_0^t \text{Cp}(t)\text{d}\tau\right] \Big/ \text{Cp}t \quad (1)$$

where Cpt is the concentration of I-Lep in serum at time t and τ is a dummy variable for time. Regression analysis was performed on the linear portion of the relation between the brain/serum ratios vs. Expt. The slope for this relation measures K_i, the blood-to-brain unidirectional influx rate.

2.3. Pretreatment and injection protocols

The time of maximal effect of a single i.p. injection of LPS (Sigma, St. Louis, MO) was first determined. Uptake of I-Lep was studied between 10 min and 72 h after an i.p. dose of LPS (3 mg/kg in 0.2 ml of LR) or immediately after (0 min) a single i.p. injection of LR solution, without LPS.

In a second study, uptake of I-Lep was studied 8 h after varying i.p. doses of LPS (0.3, 0.5, 1, 3, 5 mg/kg in 0.2 ml of LR) or LR solution without LPS (control). In another group of mice, body weights were measured before, 8, and 24 h after the i.p. injection of LR or of LPS (3 mg/kg).

2.4. Collection of cerebrospinal fluid (CSF)

Another group of mice were treated with LPS or LR and 8 h later anesthetized with urethane. The left jugular vein and the right carotid artery were exposed. The mice were given an injection into the left jugular vein of 0.2 ml of LR containing 1,000,000 cpm of I-Lep. 10 min later, the skin overlying the posterior fossa was removed and a 30-gauge needle connected to PE-10 tubing inserted into the posterior fossa to collect CSF. The exact amount in μl of CSF collected was determined by measuring the length in cm of the PE-10 tubing filled with CSF and multiplying by 0.668 (the volume of the tubing being 0.668 μl/cm). Only CSF that was absolutely clear was analysed. Blood was collected from the right carotid artery and whole brain was removed and weighed as described above. The CSF was counted for 3 h and the whole brain and serum were counted for 3 min in a gamma counter. Results are reported as brain/serum ratios and as CSF/serum ratios [(cpm/ml of CSF)/(cpm/μl of serum) = μl/ml].

2.5. Mouse serum leptin and triglycerides assays

Male ICR mice (2 months old) were injected with i.p. LR or LPS (3 mg/kg in 0.2 ml LR) and blood collected from the carotid artery 8 h later. Whole blood was

centrifuged and the serum collected and frozen at −70 °C until assay. Leptin levels in arterial serum were determined with the rodent leptin kit (Linco., St. Charles, MO). Triglyceride levels were measured on arterial serum with the kit from Sigma.

2.6. Brain perfusion

Male ICR mice (2 months old) were anesthetized with an i.p. injection of urethane 40% solution (0.2 ml). I-Lep was diluted in Zlokovic's buffer (pH 7.4; 7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l $CaCl_2$, 2.1 g/l $NaHCO_3$, 0.16 g/l KH_2PO_4, 0.17 g/l anhydrous $MgCl_2$, 0.99 g/l D-glucose, and 10 g/l BSA added on the day of perfusion). The heart was exposed in mice anesthetized with urethane by opening the thorax. The descending thoracic aorta was clamped and the right and left jugular veins severed. A 21-gauge butterfly needle was inserted into the left ventricle of heart, and the buffer containing I-Lep (250,000 cpm/ml) was infused at a rate of 2 ml/min for 1, 2, 3, 4, or 5 min. A 10-μl sample of the I-Lep buffer solution was taken from the catheter tip before insertion into the heart and used to determine the exact concentration of I-Lep perfused. After perfusion, the butterfly needle was removed, the mouse was decapitated, and the brain (minus pineal and pituitary) removed. The brain was weighed and counted in a gamma counter for 3 min. The brain/perfusate ratio (μl/g) were calculated by the formula:

$$\text{Brain/Perfusate ratio} = (\text{cpm/g of brain})/[(\text{cpm/}\mu\text{l of serum})(\text{g of brain})]. \quad (2)$$

2.7. Microvessel isolation

Male ICR mice (2 months old) were injected with LR solution without LPS (control) and with LPS (3 mg/kg in 0.2 ml LR) and blood collected from the carotid artery 8 h later. All glassware, plastics, and meshes were pre-coated with 1% BSA in LR to minimize adhesion and to maximize recovery of microvessels. Briefly, 8–9 brains from male ICR mice (2 months old) were pooled and homogenized in cold stock buffer (pH7.4; 1% dextran in Minimum Essential Medium (Gibco BRL, Grand Island, NY), 25 mM HEPES) on ice. The brains were homogenized in a glass pestle. The homogenate was then filtered through a series of nylon mesh membranes (300 μm, followed by two times 100 μm; Spectrum, Houston, TX), mixed with an equal volume of 40% dextran in stock buffer, and centrifuged at 5000 × *g* for 15 min at 4 °C. The pellet was resuspended in stock buffer and filtered through a 25 μm nylon mesh membrane (Bio-Design, Carmel, NY). The microvessels were washed from the surface of the membrane with stock buffer four times, collected, and centrifuged at 5000 × *g* for 15 min at 4 °C. Microvessel isolates were stored at −70 °C.

2.8. mRNA extraction and PCR

Microvessels stored at −80 °C had RNA extracted using RNA-STAT 60 reagent as described by the manufacturer (Tel-Test, Friendswood, TX, USA). The complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA as previously described [8]. For each group, three separate cDNAs were made and each ran in triplicate to achieve an adequate estimate of technical error. As previously described [13,22], the following primers were used for amplification of a 232 bp product for mouse Ob-Ra: upstream, 5′-acactgttaatttcacaccagag-3′; downstream, 5′-agtcattcaaaccattagtttagg-3′. To discount the possibility of confounding results with genomic DNA contamination and amplification, RNA subjected to the same procedures but lacking reverse transcriptase during the cDNA synthesis step was used as a negative control. In addition, primers were located to exons separated by at least one intron. Each 50 μl PCR reaction was performed with 5.0 μl of template cDNA. Assay conditions were 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxy(d)-NTPs, 20 pmol of each primer, 2.5 U *Taq* polymerase (Stratagene, La Jolla, CA, USA), and 0.50 μl [α-^{32}P]dCTP (New England Nuclear, Boston, MA, USA). After initial denaturation at 96 °C for 3 min, samples were subjected to 25 amplification cycles (which fell within the linear range of amplification): denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s. Five microliters of reaction product were then combined with 5 μl of sequencing stop solution (Amersham International, Aylesbury, UK) and heated to 85 °C for 5 min before loading 4 μl onto a 4% urea-acrylamide gel (38 × 31 × 0.03 cm). Electrophoresis was performed at 65 W of constant power for 3 h before the gels were transferred to filter paper, dried and finally subjected to ^{32}P quantification by PhosphoImager analysis (Molecular Dynamics, Sunnyvale, CA, USA). PCR results for Ob-Ra were normalized to β-actin mRNA levels using the following primers: Upstream: 5′-cgtaccacgggcattgtgatgg-3′; Downstream:5′-tttgatgtcacgcacgatttccc-3′ with reactions ran for 18 cycles (which fell within the linear range of amplification).

2.9. Statistical analysis

Means are reported with their standard errors. Two means were compared by Student's *t*-test. When more than two means were compared, analysis of variance (ANOVA) was performed followed by Newman–Keuls multiple comparisons test.

3. Results

Fig. 1 shows the brain/serum ratio for I-Lep between 10 min and 72 h after a single i.p. injection of LPS (3 mg/kg). LR injected immediately before study served as control (0

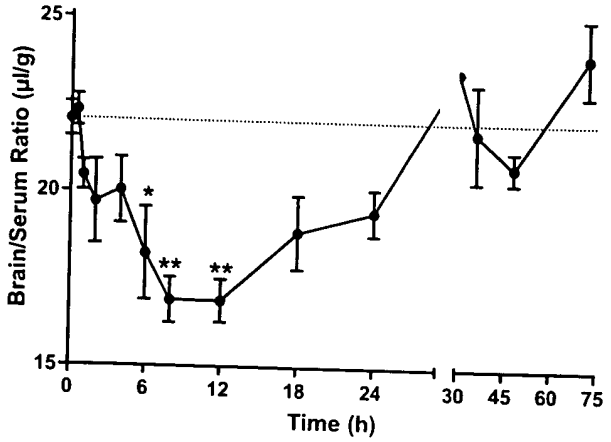

Fig. 1. The brain/serum ratio for I-Lep between 10 min and 72 h after a single i.p. injection of LPS. Uptake by brain was suppressed from 6 to 12 h after a single i.p. injection of LPS (*$p<0.05$, **$p<0.001$). The number per group averaged from 5 to 10 mice.

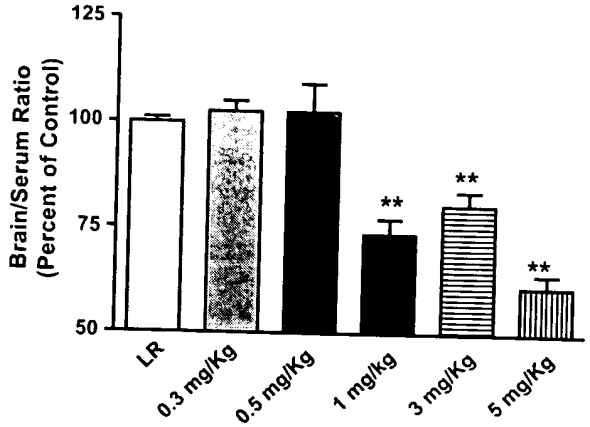

Fig. 2. Effect of LPS was dose dependent. The doses of 1, 3, and 5 mg/kg were effective (**$p<0.001$). The number per group averaged from 7 to 8 mice.

min and dotted line). ANOVA [$F(13,90)=5.83$, $p<0.0001$] showed a statistically significant effect. Uptake by brain was suppressed from 6 to 12 h after a single i.p. injection of LPS; 6 h ($p<0.05$); 8 h ($p<0.001$); 12 h ($p<0.001$).

After a single dose of LPS, mice lost weight to a statistically significant extent: [$F(5,29)=12.86$, $p<0.0001$]. The range test showed significant loss of weight at both the 8 h ($p<0.05$) and especially the 24 h ($p<0.001$) periods (Table 1).

The effect of LPS was dose dependent: [$F(5,89)=43.43$, $p<0.0001$]. A dose of 1 mg/kg reduced uptake to $73.7 \pm 10.7\%$ ($p<0.001$), 3 mg/kg reduced uptake to $80.6 \pm 10.8\%$ ($p<0.001$), and 5 mg/kg reduced uptake to $61.2 \pm 6.6\%$ ($p<0.001$). The 0.3 and 0.5 mg doses were without effect (Fig. 2).

Fig. 3 shows the brain/serum ratio (µl/g) and CSF/serum ratio (µl/ml) of I-Lep at 8 h after a single i.p. injection of LR or LPS (3 mg/kg). Results of the t-test showed the brain/serum ratio was reduced by LPS [19.73 ± 0.60 µl/g (LR) vs. 14.83 ± 0.72 µl/g (LPS), $p<0.001$], whereas the CSF/serum ratio was not [2.22 ± 0.49 µl/ml (LR) vs. 2.91 ± 0.65 µl/ml (LPS)].

To further assess the effect of LPS on leptin transport across the BBB, we employed multiple-time regression analysis. The relation between brain/serum ratios and Expt was significant for both LR ($r=0.696$, $n=10$, $p<0.001$) and LPS ($r=0.423$, $n=11$, $p<0.05$) treated mice, demonstrating that leptin transport across the BBB was measurable for both groups (Fig. 4, upper panel). The blood-to-brain unidirectional influx rate decreased by 60% from 0.906 ± 0.21 to 0.362 ± 0.16 µl/g-min, a statistically significant difference: $F(1,43)=4.37$, $p<0.05$).

There was no difference in transport of I-Lep when studied by brain perfusion 8 h after an i.p. injection of LPS (3 mg/kg). The transport rate as measured with brain perfusion was 1.23 ± 0.03 µl/g-min ($n=5$) 8 h after i.p. LR and was 1.02 ± 0.31 µl/g-min ($n=5$) 8 h after i.p. LPS (Fig. 4, lower panel).

Inclusion of LPS (1 mg/kg) in the perfusion buffer did not alter the transport of I-Lep across the BBB. The transport rate as measured with brain perfusion was 1.47 ± 0.32 µl/g-min ($n=10$) with I-Lep, and was 0.91 ± 0.30 µl/g-min ($n=10$) with I-Lep+LPS (Fig. 5). This was not a statistically significant difference (Fig. 5).

Table 1
Mouse body weight before ($t=0$), 8 h, and 24 h after a single dose of LR or LPS

Time (h)	LR (g)	LPS (g)
0	38.9 ± 0.3 ($n=5$)	39.0 ± 0.4 ($n=5$)
8	38.7 ± 0.5 ($n=5$)	36.4 ± 0.5 ($n=5$)$^{+}$
24	40.3 ± 0.8 ($n=5$)	34.9 ± 0.6 ($n=5$)*

The range test showed significant loss of weight at 8 and 24 h. $n=5$ mice/group.

*$p<0.001$ in comparison to LPS 0 h or LR 24 h.
$^{+}$ $p<0.05$ in comparison to LPS 0 h or LR 8 h.

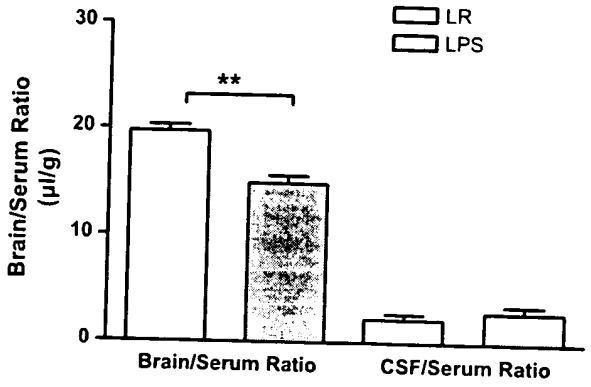

Fig. 3. The brain/serum ratio (µl/g) and CSF/serum ratio (µl/ml) of I-Lep at 8 h after a single i.p. injection of lactated Ringers solution (LR) or LPS. The brain/serum ratio was reduced by LPS: 19.73 ± 0.60 µl/g (LR) vs. 14.83 ± 0.72 µl/g (LPS) (**$p<0.001$). The CSF/serum ratio was unaffected. The number per group averaged from 6 to 9 mice.

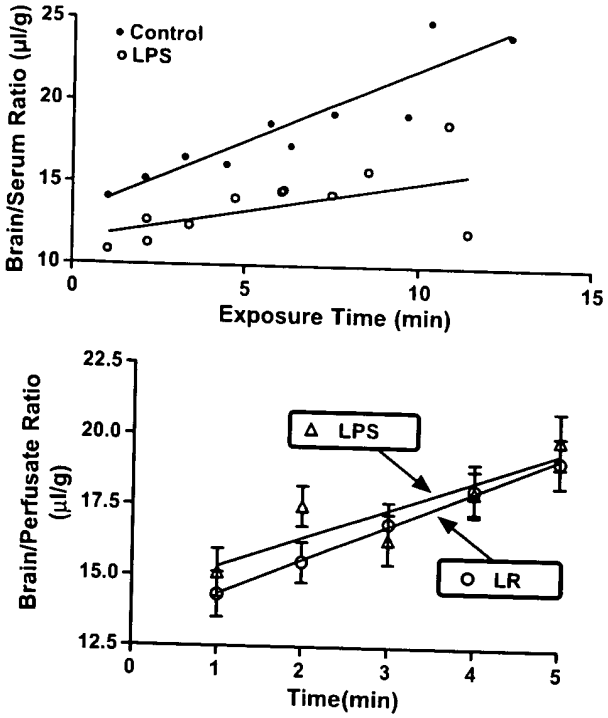

Fig. 4. The transport of I-Lep when studied by multiple-time regression analysis (upper panel) showed that LPS decreased leptin transport across the BBB by about 60% ($n=3$/time point). The transport of I-Lep when studied by brain perfusion method showed no effect of LPS treatment (lower panel). The transport rates were 1.23 ± 0.03 μl/g-min (lactated Ringers; LR), 1.02 ± 0.31 μl/g-min (LPS), $n=5$ to 7 mice/time point.

Fig. 6 shows the effect of a single i.p. injection of LPS (3 mg/kg) on levels of serum leptin (upper panel) and serum triglycerides (lower panel) 8 h after injection. Results show that serum leptin levels were significantly increased: 1.48 ± 0.25 ng/ml (LR) vs. 2.96 ± 0.57 ng/ml (LPS), $p<0.05$, $n=5$ mice/group. Serum triglycerides were increased by 44% from 48.2 ± 2.6 mg/dl ($n=6$) to 69.5 ± 7.1 mg/dl ($n=7$), $p<0.05$.

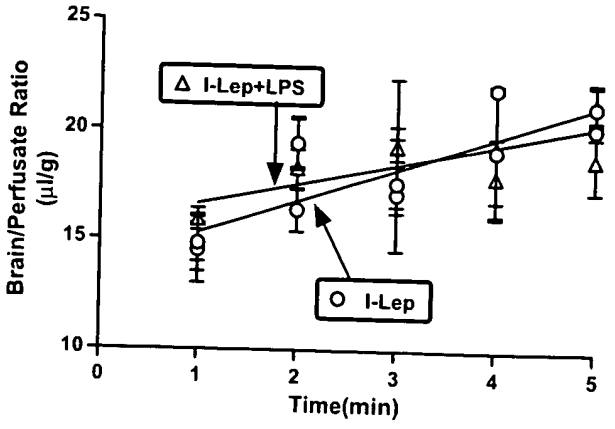

Fig. 5. The transport of I-Lep with and without the inclusion of LPS in the perfusion buffer. The transport rates were 1.47 ± 0.32 μl/g-min (I-Lep) and 0.91 ± 0.30 μl/g-min (I-Lep + LPS). There was no statistically significant difference between the two groups. The number studied was about $n=3$/cell (about 15 mice/line).

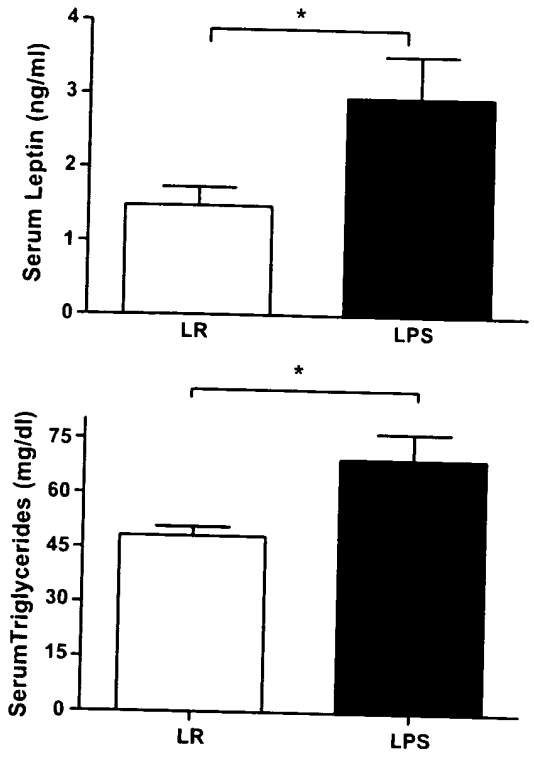

Fig. 6. Serum leptin levels (upper panel) as measured 8 h after single i.p. injection of LPS (3 mg/kg) or of LR. The serum leptin levels were 1.48 ± 0.25 ng/ml (lactated Ringers; LR) and 2.96 ± 0.57 ng/ml (LPS) ($^*p<0.05$); $n=5$ mice/group. Serum triglycerides (lower panel) were increased by 44% from 48.2 ± 2.6 to 69.5 ± 7.1 mg/dl, $p<0.05$; $n=6-7$ mice/group.

Fig. 7 shows results of the quantification of the mRNA for the Ob-Ra short isoform of leptin in isolated mouse brain microvessels. Representative RT-PCR results for Ob-Ra mRNA levels were normalized to β-Actin. No product was observed in reverse transcriptase negative controls. Ob-Ra mRNA was not altered by LPS treatment.

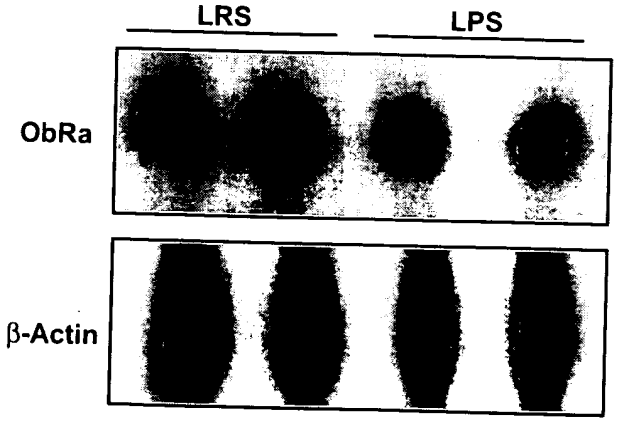

Fig. 7. RT-PCR results for Ob-Ra 8 h after i.p. LPS (3 mg/kg) or lactated Ringer's solution (LR). There was no difference in these groups after normalization with β-actin.

4. Discussion

This study investigated the effect of LPS on leptin transport across the blood–brain barrier. Previous work has shown that LPS increases leptin levels in serum [29,30]. Leptin, in turn, may mediate some of the effects of LPS. It seems well established that leptin mediates two effects of LPS: the protection against caerulein-induced pancreatitis [24] and release of interleukin-1 receptor antagonist [16,18]. Studies with leptin-deficient and leptin receptor-deficient animals have raised doubts about whether leptin may mediate LPS-induced anorexia and thermogenesis [15,23]. For leptin to mediate these effects of LPS, leptin would need to enter the brain. This raises the question of whether LPS affects the ability of leptin to cross the BBB as it does other substances [3,34]. Here, we found that the transport rate of intravenously administered, radioactively labeled leptin was suppressed from 6 to 12 h after a single i.p. injection of LPS at the dose of 3 mg/kg. This effect was most evident from 8 to 12 h after LPS injection (Fig. 1) and preceded the peak loss in body weight (Table 1). Multiple-time regression analysis has the ability to divide the brain/serum ratios into that aspect which represents transport across the BBB and that which represents non-transport components (e.g., vascular space and reversible binding to brain endothelial cells.) Analysis showed that the blood-to-brain unidirectional influx rate for I-Lep was decreased by 60%.

The effect of LPS was dose-dependent, with the 1, 3, and 5 mg/kg doses decreasing leptin transport, whereas the lower doses of 0.3 and 0.5 mg/kg had no effect (Fig. 2). In contrast to effects on the brain/serum ratio, LPS did not inhibit the CSF/serum ratio. The CSF/serum ratio was much lower than the brain/serum ratio (Fig. 3). This contrasts with the rat, which has robust leptin transport at both the vascular barrier and the choroid plexus [36]. These results suggest that leptin transport at the choroid plexus is not very robust in the mouse nor regulated in the same way as at the endothelial barrier.

Several mechanisms could explain an effect of LPS on decreased leptin transport. In considering these mechanisms, it should be noted that a decreased transport of leptin into brain would attenuate, not enhance, the signal to the CNS. The three most likely mechanisms are a direct effect of LPS on the BBB, release into the circulation of a factor which inhibits leptin transport, or an increase in serum leptin levels which would competitively inhibit I-Lep transporter. Other possibilities, such as contraction of vascular space, both would be too small to explain the reduction in I-Lep brain/serum ratios and was ruled out by multiple-time regression analysis. We used the brain perfusion method, which eliminates the immediate effect of circulants, to investigate these remaining mechanisms. Brain perfusion allows a direct assessment of the interactions between I-Lep, the BBB, and any substances added to the perfusion buffer. In contrast to I-Lep given i.v., I-Lep administered by brain perfusion had the same rate of uptake in mice administered LPS or LR. This showed that some circulating factor in blood was having an acute, immediately reversible effect on leptin transport. This substance was not LPS, as its inclusion in the perfusion buffer had no effect on I-Lep transport.

An elevation in serum leptin levels 8 h after LPS administration would provide a mechanism for the decreased transport of I-Lep seen after i.v. injection. We, therefore, measured levels of serum leptin 8 h after LPS administration and found a twofold increase (Fig. 6). Leptin levels increase linearly with adiposity, so that an obese human or mouse with a serum leptin level of 30 ng/ml will have about three times the amount of fat as a normal-weight animal with a level of 10 ng/ml [10,11,31]. Therefore, a twofold increase in serum leptin levels would be expected to be physiologically relevant.

Previous work has shown that the leptin transporter is already partially saturated at physiological levels [5]. Specifically, the level of leptin found in the serum of persons of ideal body weight approaches the Km of leptin transport. A doubling of serum levels for the range measured here would be expected to decrease brain/serum ratios about 15–20% through further saturation of the transporter. This decrease of transport efficiency means that a doubling in serum levels from 1.48 to 2.96 ng/ml would be expected to increase brain levels by about 83% [5]. However, the decrease in brain/serum ratios was not 15–20%, but 50–60%. This demonstrates that other circulating factors were also actively inhibiting leptin. Hypertriglyceridemia has recently been shown to be an important inhibitor in leptin transport across the BBB [7]. The 44% increase we saw here, although statistically significant, was modest compared to the nearly threefold increase found by others after a single injection of LPS [32]. Whether hyperleptinemia and hypertriglyceridemia are the only factors inhibiting I-Lep transport across the BBB is unknown. Overall, a decrease in transport rate exceeding 50% accompanied by a doubling of serum leptin levels would result in a net decrease in brain levels of leptin. On balance, therefore, the current analysis would tend to support those studies which cast doubt on whether the anorectic and hyperthermic effects of LPS are mediated through leptin.

LPS affected the permeability of the BBB to exogenously administered leptin through mechanisms different than those found for other substances crossing the BBB. Repeated administration of LPS can disrupt the BBB as shown by an increase in albumin uptake through a prostaglandin-dependent mechanism [34]. Additionally, transport of gp120, the viral coat of HIV-1, is increased through enhancement of adsorptive endocytosis [3]. Saturable transport of insulin is enhanced by way of a nitric oxide-sensitive, prostaglandin-independent mechanism and could explain the insulin resistance seen with bacterial infections [34].

In summary, LPS decreased the blood-to-brain transport of iv administered I-Lep competitively through the release of endogenous leptin into the circulation and also through

hypertriglyceridemia. Whereas the former mechanism would result in an overall increase in brain leptin levels, the second mechanism would tend to decrease brain levels. On balance, the decrease in the transport rate exceeds the increase in serum leptin levels. This makes it unlikely that the anorectic and hyperthermic effects of LPS are mediated through leptin.

Acknowledgements

Supported by VA Merit Review, R01 NS41863, R01 AA12743. Leptin was the kind gift of Amgen.

References

[1] W.A. Banks, Enhanced leptin transport across the blood–brain barrier by alpha1-adrenergic agents, Brain Res. 899 (2001) 209–217.
[2] W.A. Banks, A.J. Kastin, W. Huang, J.B. Jaspan, L.M. Maness, Leptin enters the brain by a saturable system independent of insulin, Peptides 17 (1996) 305–311.
[3] W.A. Banks, A.J. Kastin, J.M. Brennan, K.L. Vallance, Adsorptive endocytosis of HIV-1gp120 by blood–brain barrier is enhanced by lipopolysaccharide, Exp. Neurol. 156 (1999) 165–171.
[4] W.A. Banks, C.R. DiPalma, C.L. Farrell, Impaired transport of leptin across the blood–brain barrier in obesity, Peptides 20 (1999) 1341–1345.
[5] W.A. Banks, C.M. Clever, C.L. Farrell, Partial saturation and regional variation in the blood-to-brain transport of leptin in normal weight mice, Am. J. Physiol.: Endocrinol. Metab. 278 (2000) 1158–1165.
[6] W.A. Banks, M.L. Niehoff, D. Martin, C.L. Farrell, Leptin transport across the blood–brain barrier of the Koletsky rat is not mediated by a product of the leptin receptor gene, Brain Res. 950 (2002) 130–136.
[7] W.A. Banks, A.B. Coon, S.M. Robinson, A. Moinuddin, J.M. Shultz, R. Nakaoke, J.E. Morley, Triglycerides induce leptin resistance at the blood–brain barrier, Diabetes 53 (2004) 1253–1260.
[8] C. Bjorbaek, J.K. Elmquist, P. Michl, R.S. Ahima, A. van Beuren, A.L. McCall, J.S. Flier, Expression of leptin receptor isoforms in rat brain microvessels, Endocrinology 139 (1998) 3485–3491.
[9] L.A. Campfield, F.J. Smith, P. Burn, The OB protein (leptin) pathway—a link between adipose tissue mass and central neural networks, Horm. Metab. Res. 28 (1996) 619–632.
[10] J.F. Caro, J.W. Kolaczynski, M.R. Nyce, J.P. Ohannesian, I. Opentanova, W.H. Goldman, R.B. Lynn, P.L. Zhang, M.K. Sinha, R.V. Considine, Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance, Lancet 348 (1996) 159–161.
[11] R.V. Considine, M.K. Sinha, M.L. Heiman, A. Kriauciunas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, T.L. Bauer, J.F. Caro, Serum immunoreactive-leptin concentrations in normal-weight and obese humans, N. Engl. J. Med. 334 (1996) 292–295.
[12] R. De Matteis, S. Cinti, Ultrastructural immunolocalization of leptin receptor in mouse brain, Neuroendocrinology 68 (1998) 412–419.
[13] K. El-Haschimi, D.D. Pierroz, S.M. Hileman, C. Bjorbaek, J.S. Flier, Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesty, J. Clin. Invest. 105 (2000) 1827–1832.
[14] J.K. Elmquist, C. Bjorbaek, R.S. Ahima, J.S. Flier, C.B. Saper, Distribution of leptin receptor mRNA isoforms in rat brain, J. Comp. Neurol. 395 (1998) 535–547.
[15] R. Faggioni, J. Fuller, A. Moser, K.R. Feingold, C. Grunfeld, LPS-induced anorexia in leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice, Am. J. Physiol. 273 (1997) R181–R186.
[16] R. Faggioni, G. Fantuzzi, C. Gabay, A. Moser, C.A. Dinarello, K.R. Feingold, C. Grunfeld, Leptin deficiency enhances sensitivity to endotoxin-induced lethality, Am. J. Physiol. 276 (1999) R136–R142.
[17] B.N. Finck, R.W. Johnson, Intracerebroventricular injection of lipopolysaccharide increases plasma leptin levels, NeuroReport 10 (1999) 153–156.
[18] C. Gabay, M. Dreyer, N. Pellegrinelli, R. Chicheportiche, C.A. Meier, Leptin directly induces secretion of interleukin 1 receptor antagonist in human monocytes, J. Clin. Endocrinol. Metab. 86 (2001) 783–791.
[19] C. Grunfeld, C. Zhao, J. Fuller, A. Pollack, A. Moser, J. Friedman, K.R. Feingold, Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters, J. Clin. Invest. 97 (1996) 2152–2157.
[20] J.L. Halaas, K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Lallone, R.L. Lallone, S.K. Burley, J.M. Friedman, Weight-reducing effects of the plasma protein encoded by the obese gene, Science 269 (1995) 543–546.
[21] S.M. Hileman, J. Tornoe, J.S. Flier, C. Bjorbaek, Transcellular transport of leptin by the short leptin receptor isoform ObRa in Madin–Darby Canine Kidney cells, Endocrinology 141 (2000) 1955–1961.
[22] S.M. Hileman, D.D. Pierroz, H. Masuzaki, C. Bjorbaek, K. El-Haschimi, W.A. Banks, J.S. Flier, Characterizaton of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity, Endocrinology 143 (2002) 775–783.
[23] A.I. Ivanov, V.A. Kulchitsky, A.A. Romanovsky, Does obesity affect febrile responsiveness? Int. J. Obes. Relat. Disord.: J. Int. Assoc. Study Obes. 25 (2001) 586–589.
[24] J. Jaworek, J. Bonior, K. Nawrot, A. Leja, R. Sendur, J. Stachura, W. Pawlik, S. Konturek, Intracerebroventricular administration of bacterial lipopolysaccharide prevents the development of acute experimental pancreatitis in the rat, Med. Sci. Monit. 8 (2002) BR136–BR143.
[25] A.J. Kastin, V. Akerstrom, Fasting, but not adrenalectomy, reduces transport of leptin into the brain, Peptides 21 (2000) 679–682.
[26] S.J. Larson, A.J. Dunn, Behavioral effects of cytokines, Brain Behav. Immun. 15 (2001) 371–387.
[27] S. Laye, P. Parnet, E. Goujon, R. Dantzer, Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice, Brain Res. Mol. Brain Res. 27 (1994) 157–162.
[28] L.M. Maness, W.A. Banks, A.J. Kastin, Persistence of blood-to-brain transport of leptin in obese leptin-deficient and leptin receptor-deficient mice, Brain Res. 873 (2000) 165–167.
[29] C.V. Mastronardi, W.H. Yu, V. Rettori, S.M. McCann, Lipopolysaccharide-induced leptin release is not mediated by nitric oxide, but is blocked by dexamethasone, Neuroimmunomodulation 8 (2000) 91–97.
[30] C.V. Mastronardi, W.H. Yu, V.K. Srivastava, W.L. Dees, S.M. McCann, Lipopolysaccharide-induced leptin release is neurally controlled, Proc. Natl. Acad. Sci. U. S. A. 98 2001, pp. 14720–14725.
[31] M.A. Pelleymounter, M.J. Cullen, D. Healy, R. Hecht, D. Winters, M. McCaleb, Efficacy of exogenous recombinant murine leptin in lean and obese 10- to 12-mo-old female CD-1 mice, Am. J. Physiol. 275 (1998) R950–R959.
[32] F. Picard, S. Kupar, M. Perreault, A. Marette, Y. Deshaies, Nitric oxide mediates endotoxin-induced hypertriglyceridemia through its action on skeletal muscle lipoprotein lipase, FASEB 15 (2001) 1829–1830.
[33] B. Wispelwey, A.J. Lesse, E.J. Hansen, W.M. Scheld, Haemophilus influenzae lipopolysaccharide-induced blood–brain barrier permeability during experimental meningitis in the rat, J. Clin. Invest. 82 (1988) 1339–1346.
[34] H. Xaio, W.A. Banks, M.L. Niehoff, J.E. Morley, Effect of LPS on the

permeability of the blood–brain barrier to insulin, Brain Res. 896 (2001) 36–42.
[35] Y. Zhang, R. Proenca, Positional cloning of the mouse obese gene and its human homologue, Nature 372 (1994) 425–432.
[36] B.V. Zlokovic, S. Jovanovic, W. Maio, S. Samara, S. Verma, C.L. Farrell, Differential regulation of leptin transport by the choroid plexus and blood–brain barrier and high affinity transport systems for entry into hypothalamus and across the blood–cerebrospinal fluid barrier, Endocrinology 141 (2000) 1434–1441.